Chapter Five

Vitamin C supplementation attenuates the increases in circulating cortisol, adrenaline and anti-inflammatory polypeptides following ultramarathon running

5.1 Introduction

My colleagues and I have previously reported that vitamin C supplementation reduces the incidence of post-race upper respiratory tract infections amongst ultramarathon runners (Peters *et al.*, 1993; 1996). In a more recent study (described in chapter 4), I observed that supplementation with 1000 mg of the vitamin over an 8 day period resulted in an average 30% reduction in post-race serum cortisol levels in these athletes. I proposed that the vitamin C-associated decrease in serum cortisol might result from inhibition of enzymes involved in steroidogenesis (Pardue & Thaxton, 1984a; Ehrhart-Bornstein *et al.*, 1998; Satterlee, 1992). Alternatively, because cortisol release from the adrenals may be coupled to concomitant release of vitamin C during oxidative stress (Moser, 1992), it is possible that supplementation with the vitamin may negate the requirement for its mobilization from body stores, with a consequent, albeit secondary, attenuation of the cortisol response (Nussdorfer *et al.*, 1998). Irrespective of the biochemical mechanisms involved, the apparent vitamin C-associated attenuation of the cortisol response to strenuous exercise has potentially important implications for the prevention of transient immune dysfunction in athletes.

In the current study I have again assessed the effects of oral administration of vitamin C, albeit at different doses to those used in my previous study (chapter 4), on the increase in circulating cortisol which accompanies ultramarathon running. Moreover, I have extended my previous study to include measurements of circulating adrenaline, interleukin-10 (IL-10) and the interleukin-1 receptor antagonist (IL-1Ra).

5.2 Materials and Methods

5.2.1 Study design

Approval to conduct the study was obtained from the Human Ethics Committee of the University of Natal Medical School. Forty-five registered entrants for the 1999 Comrades Marathon signed informed consent forms. They were divided into three groups which were matched for age, gender, training status and expected race finishing time:

Group 1 (P; n =15):	Three placebo tablets per day
Group 2 (VC-500; n =15):	One 500 mg vitamin C tablet and two placebo tablets per day
Group 3 (VC-1500; n =15):	Three 500 mg vitamin C tablets per day

Subjects were blinded to their group assignment and were required to ingest one tablet with breakfast, lunch and supper over a 10 day period for 7 days preceding the race, the day of the race and for two days following the race. The vitamin C and placebo tablets were identical in appearance, taste and weight.

On the day prior to the race, subjects were required to complete 24 hour dietary records of their intake and to report for basic anthropometric measurements and blood sampling (35 ml) in the afternoon at a time which coincided with their estimated finishing time (in order to avoid the effect of diurnal rhythms on hormone concentrations). Within 30-45 minutes after completing the race, the subjects again gave 35 ml blood samples and were asked to detail their dietary and liquid intakes on the morning of the race and during the race. The blood sampling was repeated 24 hrs and 48 hrs after the race and subjects were asked to record their post-race dietary intakes for a further 36 hrs.

5.2.2 *Analysis of Dietary Records*

Intake of both food and nutritional supplements was analyzed using the Dietary Manager computer program (Program Management, Randburg, South Africa). Total

daily carbohydrate (CHO) and Vitamin C intakes during the 24 hours before, as well as on the day of the race, and after the race, including those derived from any additional carbohydrate supplements used by the athletes, were determined.

5.2.3 *Treatment of Blood*

Venous blood samples (20ml) were collected in glass Vacutainer tubes containing the anti-coagulant, tripotassium ethylenediaminetetraacetic acid (K_3-EDTA). Full blood counts were conducted on 3ml thereof. The remainder was centrifuged and the fractionated plasma quick-frozen and stored at –70°C for later analysis of Vitamins A and E, glucose, adrenaline, IL-10 and IL-1Ra. An additional 15 ml aliquot was allowed to clot at room temperature, centrifuged for 10 minutes and the serum was quick-frozen and stored at –70°C for later analysis of vitamin C and cortisol.

5.2.4 *Serum Vitamin C, Plasma Glucose, Vitamins A and E*

Vitamin C was extracted from serum using 20% trichloracetic acid and assayed using the 2,4-dinitrophenylhydrazine (Sigma Chemical Co., St Louis, MO, USA) colorimetric method (Attwood *et al.*, 1974). Plasma glucose concentrations were determined spectrophotometrically in pre-race, immediate, 24hr and 48 hr post-race samples. Plasma concentrations of vitamins A and E were determined by standard high performance liquid chromatography (HPLC) procedures following repeated (x3) extraction with hexane and using vitamin A-acetate as the internal standard (Bieri *et al.*, 1983). Quality control was maintained by inclusion of a standard consisting of pooled serum from several healthy adult human donors. With the HPLC procedures the same pool was run with all assays and the standard was extracted and assayed concurrently with all test samples.

5.2.5 *Serum Cortisol, Plasma Adrenaline, IL-10 and IL-1Ra*

Serum cortisol was assayed using the Gamma Coat radioimmunoassay procedure (Diagnostic Products Corporation, Los Angeles, CA, USA) and adrenaline using a radioimmunoassay procedure (DLD Geselschaft fur Diagnostika und medizinishe Geraete mbh, Hamburg, Germany). The plasma IL-10 and IL-1Ra analyses were part of a more comprehensive study on the cytokine profile of ultramarathon runners

which is to be described in chapter 6. These were assayed using quantitative sandwich ELISA kits provided by R&D Systems, Inc. (Minneapolis, MN, USA). A standard curve was constructed using standards provided in the kits. The assays were two step "sandwich" enzyme immunoassay procedures in which samples or standards were incubated in 96-well microtiter plates coated with polyclonal antibodies to the test cytokine as the capture antibody. Following the appropriate incubation time, the wells were washed and a second detection antibody conjugated to either alkaline phosphatase (IL-10) or horseradish peroxidase (IL-1Ra) was added. The plates were incubated and washed, and the amount of bound enzyme-labelled detection antibody was measured by adding a chromogenic substrate. The plates were then read at the appropriate wavelength (490 minus 650 nm for IL-10 and 450 minus 570 nm for IL-1Ra). The minimum detectable concentration of IL-10 was < 0.5 pg/ml and that of IL-1Ra was <22 pg/ml.

5.2.6 *Hematological analyses and adjustments*

Full blood counts were performed on K_3-EDTA treated specimens using standard hematological procedures on an automated STKS model (Coulter Electronics Inc., Hialeah, Florida, USA). Plasma volume changes were determined from pre- and post-race hemoglobin and hematocrit values using the method of Dill and Costill (1974) and subsequent post-race values (0, 24 and 48 hr) were adjusted for these plasma volume changes.

5.2.7 *Statistical Analyses*

Results are expressed as means ± SEM. An initial three-by-four repeated measures ANOVA was used to establish whether the differences between the three groups were significant throughout the 48 hr post-race period and showed that the P and VC-500 groups did not differ significantly in any of the post-race measures. These two sets of data were subsequently pooled and a further two (≤ 500mg per day vs. >1500 mg per day) -by -four repeated measures ANOVA was used to assess the group-time interaction. Wilks' Lambda trace statistic was used as the test statistic with a post-hoc correction to determine the time point of the significant differences. Statistical differences between post-race adrenaline values were determined between

≤ 500mg and >1500mg groups using Students' *t*-tests. Correlation analyses were performed using Pearson's Product Moment Correlation Coefficient. Statistical analysis was done using SAS statistical software.

5.3 Results

5.3.1 *Subjects*

Of the 45 runners recruited to the study only 29 fully complied with the protocol requirements. The characteristics of the individuals in the P, VC-500 and VC-1500 groups are shown in Table 5.1. There were no significant differences between the three groups with respect to age, height, mass, body mass index, training status, and time taken to complete the ultramarathon.

Table 5.1: Mean (±SEM) subject characteristics (n=29).

	Age (years)	**Stature (m)**	**Mass (kg)**	**BMI (kg/m^2)**	**Weekly training distance (km/wk)**	**Race time (hr)**
Placebo (n=7)	39.6 (± 2.7)	1.77 (± 0.04)	70.8 (± 4.4)	22.5 (± 1.1)	77.9(± 9.9)	9.85 (± 0.44)
VC-500 (n=10)	40.9 (± 2.9)	1.72 (± 0.02)	69.3 (± 3.4)	23.4 (± 0.9)	92.0 (± 9.8)	9.65 (± 0.36)
VC-1500 (n=12)	38.7 (± 1.5)	1.74 (± 0.02)	71.1 (± 3.4)	23.4 (± 0.7)	85.0 (± 6.7)	9.60 (± 0.22)

Table 5.2: Mean (±SEM) dietary carbohydrate (CHO) intakes and plasma concentrations of glucose and vitamins A and E on the day preceding the race and day of the race.

	CHO (g)	Plasma glucose (mmol/l)	Plasma vitamin E (μmol/l)	Plasma Vitamin A (μmol/l)
Day preceding the race				
Placebo (n=7)	399(± 29.1)	4.69 (± 0.30)	17.0 (± 1.4)	2.40 (± 0.16)
VC-500 (n=10)	499(± 51.3)	4.95 (± 0.33)	21.1 (± 1.6)	2.57 (± 0.15)
VC-1500 (n=12)	482(± 42.2)	4.74 (± 0.20)	20.7 (± 1.6)	2.29 (± 0.14)
Day of the race				
Placebo (n=7)	315 (±54.8)	6.14 (± 0.57)	16.9 (± 1.6)	2.21 (± 0.19)
VC-500 (n=10)	353(±35.2)	6.47 (± 0.51)	20.7 (± 1.5)	2.51(± 0.17)
VC-1500 (n=12)	488(±65.7)	5.95 (±0.34)	21.2 (±1.8)	2.27(±0.17)

Carbohydrate intake just prior to and during the race averaged 401(±188) g and did not differ significantly between the groups ($p>0.05$; Table 5.2). Likewise, pre- and post-race plasma glucose, vitamin A and vitamin E concentrations were not different between the 3 groups ($p>0.05$; Table 5.2). Total mean vitamin C intake on the day preceding the race (contained in supplements, beverages and foodstuffs ingested) amounted to 94.4 (±60.4), 650 (± 102) and 1603 (±90) mg in P, VC-500 and VC-1500 groups, respectively (data not shown.)

5.3.1 *Serum vitamin C*

Pre-race serum vitamin C was significantly higher in the supplemented groups by comparison with the P group (128 ± 31 and 153 ± 34 µmol/l vs 83 ± 39 µmol/l, **Figure 5.1**). Serum vitamin C concentrations were also significantly lower in placebo compared to VC-500 and VC-1500 groups at the 24 h post-race and 48 h post-race time points (**Figure 5.1**). There was a significant increase ($\bar{X}$ = 42.6 µmol/l) in serum vitamin C in the P group immediately post-race ($p<0.05$).

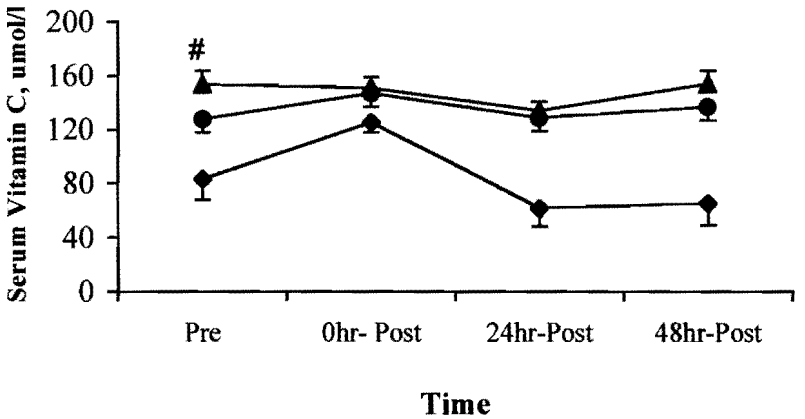

Figure 5.1: Pattern of change in mean serum vitamin C concentrations before and after the 1999 Comrades 90 km ultramarathon in ♦ placebo, ● VC-500 and ▲ VC-1500 groups. Data presented as means ±SEM. Time effect: $p<0.001$; group vs time interaction effect: $p=0.27$, group effect: $p=0.006$ # $p<0.05$ Bonferroni multiple comparison test between ≤500 and >1500mg groups at time point.

This increase in the mean serum vitamin C was attenuated in both of the vitamin supplemented groups (19.3 and -2.84 µmol/l in VC-500 and VC-1500 groups, respectively). At 24 and 48 hrs after completion of the race the serum vitamin C concentrations returned to values which were not significantly different ($p>0.05$) from pre-race values.

Table 5. 3: Hematological profile. Values as mean (±SEM).

Variable	Pre-race	Post-race (0.5-1hr)	Post-race (24 hours)	Post race (48 hours)	Time effect; Interaction effect; Group effect**
Packed cell volume (%)					
Placebo	41.5 (± 2.7)	44.0 (±2.3)	41.6 (± 1.1)	39.4 (± 1.0)	P<0.001
VC-500	42.6 (± 0.7)	43.0 (±0.8)	40.2 (± 0.9)	39.6 (± 0.8)	P=1.10
VC-1500	42.0 (± 0.7)	44.2 (± 1.2)	40.4 (± 1.0)	38.3 (±0.8)	P=0.62
Hemoglobin (g/l)					
Placebo	141 (± 4.5)	146 (± 4.9)	144 (± 3.8)	134 (± 4.5)	P<0.001
VC-500	143 (± 2.5)	145 (± 2.9)	139 (± 3.5)	135 (± 2.9)	P=1.12
VC-1500	142 (± 2.6)	146 (± 3.8)	137 (± 3.2)	130 (± 2.3)	P=0.63
% PV change *					
Placebo		-7.1 (± 3.0)	-2.2 (± 3.6)	9.5 (± 2.5)	P<0.001
VC-500		-1.7 (± 1.8)	7.7 (± 2.3)	12.3 (±2.3)	P=0.11
VC-1500		-6.7 (± 2.2)	5.4 (± 2.6)	15.6 (±2.3)	P=0.63
Total leukocytes(10^9/l)					
Placebo	7.6 (± 1.1)	18.1 (± 2.5)	8.7 (± 1.0)	8.1 (± 1.0)	P<0.001
VC-500	8.0 (± 1.1)	16.6 (± 1.2)	9.4 (± 0.7)	7.8 (± 0.3)	P=0.20
VC-1500	6.5 (± 0.5)	14.2 (± 1.1)	8.1 (± 0.7)	7.2 (± 0.5)	P=0.07
Neutrophils (10^9/l)					
P Group	4.4 (± 0.9)	15.2 (± 2.2)	5.1 (± 0.1)	4.0 (± 0.8)	P<0.001
VC- 500	4.8 (± 1.1)	13,8 (± 0.9)	5.6 (± 0.6)	3.6 (± 0.3)	P=0.10
VC-1500	3.5 (± 0.4)#	11.0(± 1.0)#	4.4 (± 0.1)	3.1 (± 0.2)	P=0.03
Lymphocytes (10^9/l)					
P Group	2.1 (± 0.1)	1.6 (± 0.3)	2.4 (± 0.3)	2.3 (± 0.3)	P<0.001
VC- 500	2.2 (± 0.1)	1.3 (± 0.1)	2.6 (± 0.2)	2.2 (± 0.1)	P=0.95
VC-1500	2.3 (± 0.2)	2.0 (± 0.3#	2.7 (± 0.2)	2.4 (± 0.2)	P=0.22
Neutro:Lymph ratio					
P Group	2.1 (± 0.4)	11.9 (± 2.3)	2.5 (± 1.5)	2.2 (± 1.1)	P<0.001
VC- 500	2.3 (± 0.6)	10.7 (± 1.3)	2.4 (± 0.9)	1.7 (± 0.5)	P=0.08
VC-1500	1.5 (± 0.3)	7.0 (± 1.9)#	2.0 (± 1.5)	1.5 (± 0.8)	P=0.02

*relative to pre-race plasma volume; PV= plasma volume; neutro:lymph ratio=neutrophil :lymphocyte ratio ** Repeated measures ANOVA # p<0.05 Bonferroni multiple comparison test between groups at time point when compared to ≤ 500mg group.

5.3.2 *Blood counts*

Results of the full blood counts are shown in Table 5.3. Packed cell volume and hemoglobin values indicated a varied hydration status with 27.5% presenting with an increase in plasma volume immediately following participation in the ultramarathon. The difference in plasma volume did not differ significantly between the groups.

Significant immediate post-race lymphopenia and neutrophilia was present in all 3 groups with recovery to normal values at 24 and 48 hrs after completion of the race. The smaller relative magnitude of the lymphopenia and neutrophilia, as expressed in the neutrophil:lymphocyte ratio, in the VC-1500 group (n=12) in comparison to the ≤ VC-500 group (n=17) also reached statistical significance ($p<0.05$) over the 48 hour post-race period.

5.3.2 *Circulating cortisol, adrenaline, IL-10 and IL-1Ra concentrations*

Circulating cortisol and adrenaline increased significantly in all 3 groups immediately post-race, subsiding to close to pre-race values (in the case of cortisol) at 24 and 48 hrs after completion of the race (Table 5.4). The increase in both cortisol and adrenaline observed immediately post-race was attenuated in the VC-1500 group relative to the ≤ 500mg groups ($p<0.001$ and $p<0.05$, respectively). Pre-race adrenaline levels were also less in the VC-1500 group ($p<0.05$). The immediate post-race values for IL-10 and IL-1Ra were significantly higher in relation to the pre-race values and subsided to close to pre-race values at 24 and 48 hrs after completion of the race (Table 5.4). However, the increase in the circulating concentrations of these anti-inflammatory polypeptides observed immediately post-race was significantly attentuated in the VC-1500 group ($p=0.05$) when compared to the ≤ 500mg groups.

Correlation analyses between collective data pooled for all subjects (n=124) revealed a significant positive correlation between serum cortisol and IL-10 ($r=0.79$) and inverse correlation between pre-race vitamin C values and post-race serum cortisol ($r=-0.30$; $p<0.05$) Significant ($p<0.05$) correlations were found between

post-race serum cortisol and both IL-10 (r=0.61) and IL-1Ra (r=0.50) as well as adrenaline and IL-1Ra (r=0.71).

Table 5.4: Mean (±SEM) stress hormone and anti-inflammatory polypeptide concentrations

	Pre-race	Post-race** (0.5-1hr)	Post-race** (24 hours)	Post race** (48 hours)	Time effect; Interaction effect; Group effect***
Serum Cortisol (nmol/l)					
P Group	347 (± 41.5)	1179 (± 93.2)	323 (± 59.6)	329 (± 70.9)	P<0.001
VC- 500	260 (± 33.5)	1205 (± 97.5)	300 (± 26.3)	284 (± 27.1)	P= 0.003
VC-1500	248 (± 32.9)	770 (± 64.7)#	262 (± 21.8)	329 (± 46.5)	P= 0.02
Plasma Adrenaline (pg/ml)					
P Group	93.4 (± 16.7)	204 (± 48.7)			
VC- 500	140 (± 49.1)	257 (± 59.4)	ND	ND	
VC-1500	56.0(± 8.83)*	120 (± 21.5)*			
Plasma IL-10 (pg/ml)					
P Group	0.46 (± 0.26)	83.1 (± 22.9)	0.61 (± 0.23)	0.62 (± 0.30)	P<0.001
VC- 500	0.56 (± 0.24)	69.6 (± 18.5)	0.91 (± 0.34)	0.80 (± 0.27)	P=0.001
VC-1500	0.35 (± 0.15)	31.5 (± 8.81)#	0.39 (± 0.14)	0.30 (± 0.07)	P=0.01
Plasma IL-1Ra (pg/ml)					
P Group	176 (± 21.8)	2850(± 1084)	320(± 29.4)	249 (± 23.8)	P<0.001
VC-500	184 (± 24.8)	4241(± 1051)	439 (± 63.6)	327 (± 56.3)	P=0. 07
VC-1500	193 (± 15.5)	1519(± 434)#	282 (± 24.7)	288 (± 25.1)	P=0.04

# p<0.05 Bonferroni multiple comparison test between groups at time point; * p<0.05 vs ≤500 group; Students t-test, adjusted for base-line values; **adjusted for plasma volume changes from pre-race ;*** Repeated measures ANOVA; ND = not done

5.4 Discussion

The increase in circulating concentrations of the adrenal immunosuppressive, anti-inflammatory hormones cortisol, adrenaline and noradrenaline, which accompanies intensive physical exercise is well-documented (Keast *et al.*, 1988; Skinkai *et al.*, 1996, Suzuki *et al.*, 1999). An earlier laboratory study (Nieman *et al.*, 1997) on 6 pairs of runners failed to report an effect of vitamin C supplementation on immune

response to 2.5 hours of treadmill running. I have, however, reported that 1000mg vitamin C supplementation (total mean intake: 1339 mg /day) in ultramarathoners is associated with attenuation of the increase in serum cortisol observed immediately post-race following an ultramarathon lasting 9 -11 hours (Chapter 4). In the current study I have investigated the effects of vitamin C supplementation, at different doses (500 mg and 1500 mg/daily) to those used in my previous study (Chapter 4), on the cortisol response which accompanies participation in the same 90 km ultramarathon and included measurements of circulating adrenaline and those of the anti-inflammatory polypeptides, IL-10 and the IL-1Ra in an extension of this study.

As previously reported by myself (chapter 4) and others (Gleeson *et al.*, 1987; Brites *et al.*, 1999), vitamin C levels were increased in the placebo group on completion of the ultramarathon and subsided at 24 and 48 hrs thereafter. This apparent mobilization of vitamin C appears to represent an adaptive response to exercise-induced oxidative stress (Brites *et al.*, 1999). Pre-race serum vitamin C values and those measured 24 and 48 hrs after completion of the race were also significantly higher in the vitamin-supplemented group than those of the placebo groups. Interestingly, the difference in serum vitamin C between the placebo (51.4 % higher than the pre-race value) and vitamin-supplemented groups was considerably less and statistically insignificant immediately post-race. The corresponding average changes in circulating vitamin C concentrations in the immediate post-race VC-500 and VC-1500 groups were 13.0 % and -0.02% respectively. These observations confirm my previous findings (described in chapter 4) that supplementation with vitamin C appears to negate the requirement for mobilization of the vitamin from the adrenal gland and other body storage sites during intensive physical stress (Borish, 1998).

Somewhat surprisingly the pre-race serum vitamin C levels observed following supplementation with 500 mg daily of the vitamin in the current study (Chapter 5, Figure 5.1) were similar to those observed following supplementation with 1000 mg daily of the vitamin described in the previous chapter (Table 4.3). However, the differences observed between these two supplementation regimens with respect to

mobilization of the vitamin following intensive exercise, as well as the effects on circulating cortisol, suggest that pre-race serum concentrations of the vitamin at these doses may not necessarily reflect tissue concentrations.

In agreement with my previous study (chapter 4), administration of vitamin C at 1500 mg/daily, but not at 500 mg/daily, significantly attenuated (average decrease of 34.7% relative to P group) the immediate post-race increase in serum cortisol. Pre-race concentrations of serum cortisol, as well as those measured at 24 and 48 hrs after completion of the ultramarathon event, were somewhat lower, although not significantly so, in both vitamin-supplemented groups relative to the P group. These observations are also in agreement with a recent report in which administration of vitamin C (1000 mg/daily) in combination with vitamin E to healthy, elderly humans was accompanied by a significant decrease in serum cortisol and improved immune function (De la Fuente *et al.,* 1998) and confirm previous findings on animals (Pardue & Thaxton, 1985a; Satterlee, 1989; 1994; Enwonuwo *et al.,* 1995; Jones *et al.,* 1999).

Although blood sampling for adrenaline concentrations should ideally have been performed immediately on completion of the race, this was not logistically possible in a competitive event of this nature. It is, however, noteworthy, that circulatory adrenaline concentrations were reduced significantly following a week of supplementation with vitamin C both prior to and following the stressful competitive event when compared to those of the unsupplemented runners. The average decreases relative to the P group were of 40% and 41% respectively in the group of athletes supplemented with 1500 mg vitamin C daily, but were not significantly lower ($p<0.05$) in those supplemented with $\leq$ 500 mg/daily vitamin C.

It is possible that the observed vitamin C-related attenuation of the exercise-induced increase in circulating cortisol and adrenaline may, in part, explain the reported decrease in the incidence of upper respiratory infections in vitamin C-supplemented ultramarathon athletes. Both of these adrenal hormones possess potent anti-inflammatory, immunosuppressive properties and may impact on the magnitude of

the post-exercise "open-window" period (Pedersen & Ullum, 1994) with a delayed manifestation of actual symptoms of infection following varying incubation periods. Corticosteroids have been shown to mediate these immunomodulatory actions by interaction with cytosolic glucocorticoid receptors (Barnes & Adcock, 1993; Barnes & Karin, 1997; Rahman & MacNee, 1998), while adrenaline operates via cyclic AMP-coupled $ß_2$-adrenoreceptors on immune and inflammatory cells (Moore & Willoughby, 1995; Van der Poll *et al.*, 1996; Weiss *et al.*, 1996).

The proposed relationship between vitamin C-associated suppression of cortisol release from the adrenals and possible potentiation of immune function, is further strengthened by the observation that the dramatic increase in the circulating concentration of the broad-spectrum anti-inflammatory cytokine, IL-10 (Borish, 1998), observed immediately after completion of the ultramarathon event, was significantly attenuated in the group of athletes supplemented with 1500 mg/daily of the vitamin. This is also supported by the coefficient of correlation of 0.79 between the circulating concentrations of cortisol and IL-10 obtained from the findings of this study. Production of IL-10 by immune and inflammatory cells is potentiated by corticosteroids (Suzuki *et al.,* 1999) and adrenaline (Van der Poll *et al.*, 1996). Interleukin-10, in turn, acts on monocytes/macrophages to stimulate release of IL1-Ra (Borish, 1998), an endogenous antagonist of the pro-inflammatory cytokine, IL-1. Interestingly it has recently been reported that rhinoviruses, the predominant cause of the common cold, increase the production of IL-10 by monocytes, suggesting that increased levels of this cytokine may contribute to the pathogenesis of infection with these viral pathogens (Stöckl *et al.,* 1999). It is therefore possible, but not proven, that vitamin C supplementation, through attenuation of the cortisol, adrenaline and IL-10 responses which accompany intensive exercise, may contribute towards the prevention of the resultant transient immunosuppression which predisposes to upper respiratory tract infections (Peters *et al.,* 1993; 1996).

The biochemical mechanisms by which vitamin C supplementation attenuates the adrenal hormone response to exercise-induced oxidative stress remains to be established. However, my observation that the release of both cortisol and

adrenaline is attenuated by supplementation with the vitamin appears to favor a mechanism by which the release of these anti-inflammatory hormones is coupled to mobilization of vitamin C from the adrenals (Moser, 1992), as opposed to inhibitory effects of the vitamin on the synthesis of these hormones (Pardue & Thaxton, 1984a; Satterlee, 1992). Oxidative stress is presumably the trigger for the combined release of vitamin C, cortisol and adrenaline from the adrenals, with all three cooperating to protect against inflammation-mediated tissue damage.

In conclusion, oral supplementation with vitamin C at 1500 mg daily attenuated the increases in the production of the immunosuppressive adrenal hormones, cortisol and adrenaline, which accompanies intensive exercise, as well as the production of the anti-inflammatory polypeptides IL-10 and IL-1Ra. The findings of this study did not, however, reveal a linear dose-dependent response. Instead, the combined results of this work and my previous studies (Peters *et al.*, 1993; 1996; chapter 4) in which total vitamin C ingestion varied from 1139 and 1004 mg/day, respectively in the early studies (Peters *et al.*, 1993, 1996) and 1339 mg /day in my most recent work (chapter 4) appear to point towards a threshold value existing at approximately 1000 mg per day. As it is possible, however, that inhibitory effects of a vitamin C intake of 650 mg daily do indeed occur, but are only evident at earlier time-points during the race, as opposed to on its completion, the relationship, if any, between these immunomodulatory effects of a daily dosage ranging from 650-1603 mg vitamin C and the possible protective effects of this vitamin against post-exercise upper respiratory tract infection, do require further investigation.

This chapter limited itself to a selected set of related findings which confirm the initial finding of an attenuation of the exercise-induced elevation of cortisol described in the previous study in chapter 4. A more comprehensive description of the cytokine profile of this group of runners is presented in chapter 6.

Chapter Six

Influence of vitamin C supplementation on cytokine changes following an ultramarathon

6.1 Introduction

Vitamin C (ascorbate) is a water-soluble vitamin present in the cytosolic compartment of the cell and the extracellular fluid. Of all essential nutrients, vitamin C has generated the greatest interest for its potential influence on host defense mechanisms and the immune system (Hughes, 1999). The concentration of vitamin C is unusually high in activated neutrophils and macrophages (Washko *et al.*, 1991; Wolf, 1993). Supplemental amounts of vitamin C have been shown to alter many different indices of human immune responses (Anderson *et al.*, 1980; Jacob *et al.*, 1991; Campbell *et al.*, 1999). Vitamin C also provides *in vivo* antioxidant protection primarily as an aqueous-phase peroxyl and oxygen radical scavenger, and is concentrated in those tissues and fluids which have a high potential for radical generation (Jacob & Burri, 1996). Vitamin C exerts a protective effect on neutrophil-mediated cell injury by scavenging reactive oxygen metabolites following physical trauma (Dwenger *et al.*, 1992; Jonas *et al.*, 1993). Free radical-mediated processes appear to be an important component of exercise-induced muscle and lymphoid tissue damage and inflammation (Azenabor & Hoffman-Goetz, 1999; Goldfarb, 1999). Numerous recent studies have indicated that vitamin C supplementation attenuates exercise-induced oxidative stress (Sanchez-Quesada *et al.*, 1998; Vasankari *et al.*, 1998; Ashton *et al.*, 1999: Schröder *et al.*, 2000).

The concentration of vitamin C in the adrenal cortex is higher than in any other organ (Redmann et al., 1995). Although poorly defined in humans, vitamin C depletion or supplementation appears to alter serum cortisol levels in some animal models (Kodama *et al.*, 1994; Satterlee *et al.*, 1994; Enwonwu *et al.*, 1995; Redmann *et al.*, 1995; Jones *et al.*, 1999). This may be important in view of the well-defined role of cortisol in leukocyte trafficking and function following heavy

exertion as well as the well described immuno-suppressive actions of cortisol (Cupps & Fauci, 1982)

Given the importance of vitamin C to the immune system, the effect of supplemental amounts of this nutrient in altering immune function and quenching the reactivity of exercise-induced free radicals, and its potential role in altering serum cortisol levels, a randomized, double-blind, placebo-controlled study was designed to investigate the influence of supplemental vitamin C on the immune response to 2.5 hours of treadmill running by 12 marathoners (Nieman *et al*, 1997). Vitamin C compared to placebo supplementation (1000 mg/day for 8 days) had no significant effect on the pattern of change in cortisol, IL-6, or other immune measures following the exercise bout. This study, however, had a small number of subjects (six in each group), induced a relatively low degree of physiologic and oxidative stress, and the carbohydrate intake of the subjects, which has been shown to significantly affect the parameters measured (Nehlsen-Cannarella *et al.*, 1997; Nieman *et al*, 1998), was not controlled. A subsequent field study performed on ultramarathoners showed an attenuation of the post-exercise cortisol response in runners supplemented with vitamin C (Chapter 4). As a follow-up and extension of these studies, I investigated the influence of vitamin C supplementation at two levels (500 and 1,500 mg/day) on the pattern of change in concentration of serum cortisol and plasma cytokines in runners following a competitive ultramarathon.

6.2 Materials and Methods

The Comrades Marathon is a 90 km race event held each year during the winter in South Africa. Twenty-nine entrants to the 1999 Comrades Marathon (same as in chapter 5) volunteered to be subjects in this study, and complied with all aspects of the research design. Subject selection criteria included: 1) non-smoking; 2) no clinical signs of infection; 3) no intake of analgesic or anti-inflammatory medication prior to and during the race; 4) no regular use of vitamin C supplements; and 5) willingness to adhere to all aspects of the study design. The protocol was approved

by the Human Ethics Committee of the University of Natal Medical School and informed consent was obtained from subject.

6.2.1 *Research Design*

The study was based on a 3 (three groups) by 4 (four blood samples) repeated measures design. Subjects were divided into three groups:

Group 1 (Placebo): Placebo supplement (three placebo tablets per day).
Group 2 (VC-500): One 500 mg vitamin C tablet and two placebo tablets per day.
Group 3 (VC-1500): Three 500 mg vitamin C tablets per day.

Each subject in each group received three tablets per day, with one tablet ingested with breakfast, lunch, and supper. The vitamin C and placebo tablets were identical in appearance, taste, and weight, and subjects were blinded to their group assignment. Subjects ingested the supplements for seven days prior to the race, on race day, and for two days after (10 days total). Diet and fluid intake was recorded on the morning of and during the 90 km event, with carbohydrate intake determined through use of the Dietary Manager software program (Program Management, Randburg, South Africa).

On the afternoon preceding the race (about 14-16 h pre-race), height, body mass, age, and training history were recorded.

6.2.2 *Blood Samples and Assays*

A 30 ml venous blood sample was also collected on the afternoon preceding the race (14-16 h prior to the race). Post-race venous blood samples were collected within 30-45 min after completion of the race event, and then again 24 h and 48 h post-race. Plasma and serum aliquots were stored at -70°C, and analyzed for cortisol, vitamin C, and cytokine concentrations. The serum vitamin C, cortisol, IL-10 and IL-Ra data are part of a study examining the effects of vitamin C supplementation

on indices of immune function following ultramarathon running. This paper, however, focuses on the plasma cytokine data from this larger study.

Serum cortisol was assayed using a competitive solid-phase ^{125}I radioimmunoassay (RIA) technique (Diagnostic Products Corporation, Los Angeles CA). Serum vitamin C was extracted from the serum using 20% trichloracetic acid and assayed using the 2,4-dinitrophenylhydrazine colorimetric method (Sigma Chemical Co., St Louis, MO, USA).

Total plasma concentrations of interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), interleukin-1 receptor antagonist (IL-1Ra), and tumor necrosis factor-α (TNFα) were determined using quantitative sandwich ELISA kits provided by R&D Systems, Inc. (Minneapolis, MN). A standard curve was constructed using standards provided in the kits and the cytokine concentrations were determined from the standard curves using linear regression analysis. The assays were two step "sandwich" enzyme immunoassays in which samples and standards were incubated in a 96-well microtiter plate coated with polyclonal antibodies for the test cytokine as the capture antibody. Following the appropriate incubation time, the wells were washed and a second detection antibody conjugated to either alkaline phosphatase (IL-1β, IL-6, IL-10, TNFα) or horseradish peroxidase (IL-8, IL-1Ra) was added. The plates were incubated and washed, and the amount of bound enzyme-labeled detection antibody was measured by adding a chromogenic substrate. The plates were then read at the appropriate wavelength (490 minus 650 nm for IL-1β, IL-6, IL-10, and TNFα; 450 minus 570 nm for IL-8 and IL-1ra). The minimum detectable concentration of IL-1β was <0.1 pg/ml, of IL-6 was <0.094 pg/ml, of IL-8 was <10 pg/ml, of IL-10 was < 0.5 pg/ml, of IL-1Ra was <22 pg/ml, and of TNFα was 0.18 pg/ml.

All post-race blood data reported in this chapter were adjusted for plasma volume changes. These were calculated from pre- and post-race hematocrit and hemoglobin differences (given in chapter 5)

6.2.3 *Data Analysis*

Results are expressed as mean ± SEM. A three by four repeated measures ANOVA with two between-subjects factors (placebo, VC- 500, VC-1500) and one within-subjects factor (time of measurement) was used to analyze the data. When Box's M suggested that the assumptions necessary for the univariate approach were not tenable, the multivariate approach to repeated measures was used. In the latter case, Pillais trace statistic was used as the test statistic. When the group x time interaction p-value was ≤ 0.05, the Tukey multiple comparison test was used to compare groups at a particular time point. When it was determined that the placebo and VC- 500 groups did not differ in any of the post-race measures, the data were reanalyzed using a two (placebo and VC- 500 combined compared to VC-1500) by four repeated measures ANOVA. Pearson correlations were used to test the association between serum cortisol and plasma cytokine concentrations post-race.

6.3 *Results*

Twenty-nine subjects ranging in age from 27 to 54 years fully complied with all protocol requirements (Table 6.1). This included 7 in the placebo group, 10 in the VC-500 group, and 12 in the VC-1500 group. Age, body mass, and stature did not differ significantly between groups. Serum vitamin C concentrations were significantly lower in the placebo compared to VC-500 and VC-1500 groups at the pre-race, 24 h post-race, and 48 h post-race time points (Table 6.2).

Table 6.1 Subject Characteristics (n=29)

Characteristic	Mean (±SEM)	Range
Age (yr)	39.7(±1.30)	27.5-54.0
Body Mass (kg)	70.4(±2.04)	53.2-97.0
Stature (m)	1.74 (±0.02)	1.57-1.89
Body mass index (kg/m^2)	23.2 (±0.50)	18.7 –28.7
Race Time (hrs)	9.73 (±0.18)	7.38-11.08
Weekly Training distance (km)	87.9 (± 4.92)	70.0-120

Serum cortisol increased in all groups immediately following the race, but significantly less so in the VC-1500 group (**Figure 6.1** and Table 6.2).

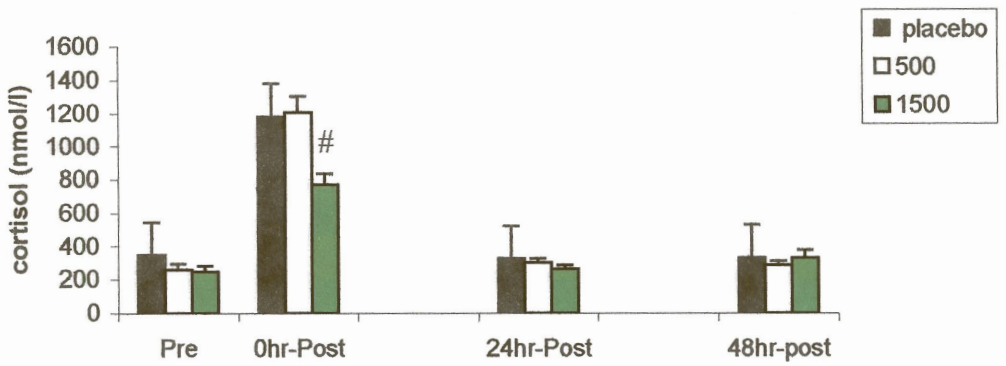

Figure 6.1 Serum cortisol concentrations before and after the 1999 Comrades 90 km ultramarathon in placebo, VC-500, and VC-1500 groups. Data presented as mean ±SEM. # $p<0.01$, Tukey multiple comparison test between groups (VC-1500 and ≤500mg) at time point.

All measured plasma cytokine concentrations were significantly elevated immediately post-race, with the magnitude of increase for TNF-α and IL-1β much smaller than for IL-6, IL-8, IL-10 and IL-1ra (**Figures 6.2-6.5**, Table 6.3). IL-10, IL-8, TNF-α, and IL-1β returned to pre-race levels within 24 hours, whereas IL-6 and IL1Ra dropped back to resting levels with 48 hours.

Group x time interaction statistics between the three groups were not significant for any of the plasma cytokines. However, when the placebo and VC-500 groups were combined (N=17) and compared to VC-1500 (N=12), immediate post-race plasma concentrations were significantly lower in the VC-1500 group for IL-1Ra (-57%) and IL-10 (-57%), with a trend measured for IL-6 (-27%, P=0.11) and IL-8 (-26%, P=0.14) (**Figures 6.2-6.6).** Table 6.2 presents the differences between the VC-1500 *and ≤500mg Vit C in immediate post-race data.*

Table 6.2: Mean ±SEM immediate post-race cortisol and cytokine concentrations in ≤500mg Vit C and ≥ 1500 mg Vit C groups

	≤500 (n=17)	≥1500 (n=12)	Significance*
Serum Cortisol (nmol/l)	1194 ± 95	770± 64.7#	p<0.01
Plasma IL-Ra	3668 ± 1064	1519± 434#	P=0.04
Plasma IL-10	75.2 ± 20.3	31.5± 8.81#	p= 0.01
Plasma IL-6	108± 20.2	78.4 ± 5.8	p=0.11
Plasma IL-8	27.1 ± 5.4	20.9 ± 2.3	p=0.14

* Tukey multiple comparison test between two groups at time point # p<0.05

Table 6.3 : Serum vitamin C, and plasma IL-1β and TNF-α concentrations before and after the 1999 Comrades 90K ultramarathon. Placebo group, n=7; 500 mg/d vitamin C supplement group (VC-500), n=10; 1500 mg/d vitamin C supplement group (VC-1500), n=12. Data presented as mean ±SE.M

Parameter	Pre-race	Post-race (0.5-1 hr)	Post-race (24-hrs)	Post-race (48-hrs)	Time effect; Interaction effect
Vitamin C (µmol/l)					**p<0.001**
Placebo	82.9 ±10.8	125.5 ±6.5	61.3 ±6.8	65.2 ±11.9	**p=0.065**
VC-500	127.8 ±10.2*	146.9 ±12.3	128.9 ±7.9*	136.9 ±9.7*	
VC-1500	153.4 ±10.2*	150.9 ±7.8	134.6 ±7.4*	153.4 ±8.5*	
TNF-α (pg/ml)					**p<0.001**
Placebo	5.86 ±0.77	6.46 ±1.10	4.70 ±0.62	4.90 ±0.84	**p=0.419**
VC-500	7.45 ±1.47	10.24 ±2.17	7.20 ±1.14	6.83 ±1.30	
VC-1500	4.63 ±0.66	6.63 ±0.70	5.05 ±0.43	4.37 ±0.48	
IL-1β(pg/ml)					**p<0.001**
Placebo	0.22 ±0.10	0.44 ±0.11	0.14 ±0.07	0.14 ±0.07	**p=0.124**
VC-500	0.13 ±0.05	0.55 ±0.15	0.11 ±0.04	0.20 ±0.09	
VC-1500	0.04 ±0.01	0.29 ±0.06	0.06 ±0.01	0.02 ±0.01	

* p<0.05, Tukey multiple comparison test between two groups (≤500mg Vit C and ≥ 1500 mg Vit C) at time point

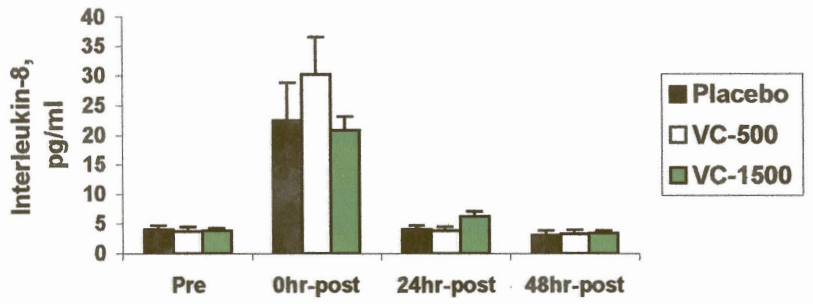

Figure 6.2: Plasma IL-8 concentrations before and after the ultramarathon in placebo, VC-500, and VC-1500 groups. Data presented as mean ±SEM.

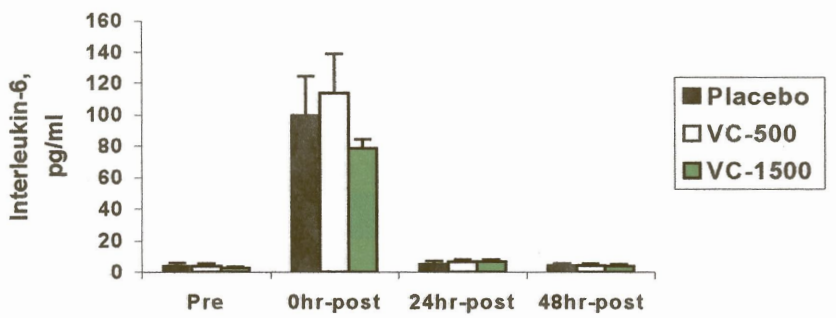

Figure 6.3 Plasma IL-6 concentrations before and after the ultramarathon in placebo, VC-500, and VC-1500 groups. Data presented as mean ±SEM

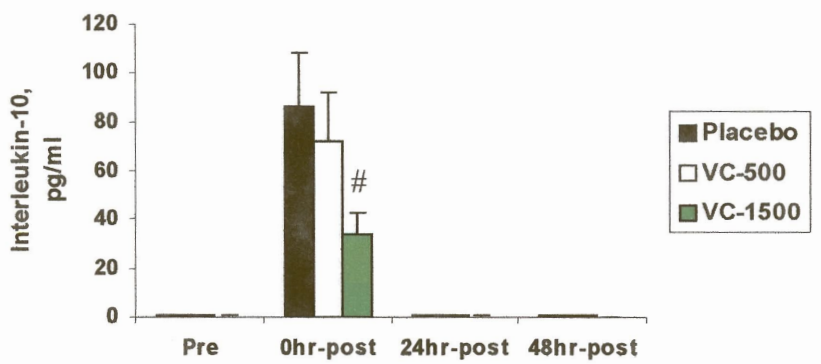

Figure 6.4: Plasma IL-10 concentrations before and after the ultramarathon in placebo, VC-500, and VC-1500 groups. Data presented as mean ±SEM; #$p<0.05$, Tukey multiple comparison test between groups at time point.

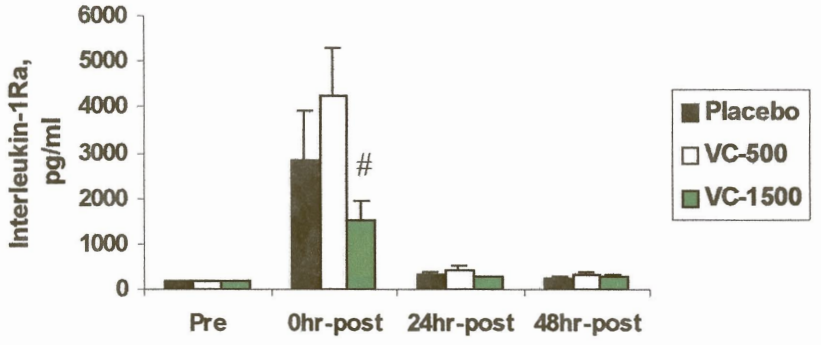

Figure 6.5: Plasma IL-1Ra concentrations before and after the ultramarathon in placebo, VC-500, and VC-1500 groups. Data presented as mean ±SEM; #p<0.05, Tukey multiple comparison test between groups at time point.

For all subjects combined, pre-race serum vitamin C concentrations were negatively correlated with post-race cortisol concentrations (r=-0.33, p=0.08). Post-race cortisol concentrations were significantly correlated with post-race IL-10 (r=0.65, p<0.01), IL-6 (r=0.47, p=0.01), IL-1β (r=0.43, p=0.02), and IL-1ra (r=0.42, p=0.03). When pre- and post-race data were pooled (N=124), serum cortisol concentrations were significantly correlated with IL-6 (r-0.83), IL-10 (r=0.79), IL-8 (r=0.78), IL-1Ra (r=0.69), IL-1B(r=0.57). These data are presented in **Figures 6.6-6.11**.

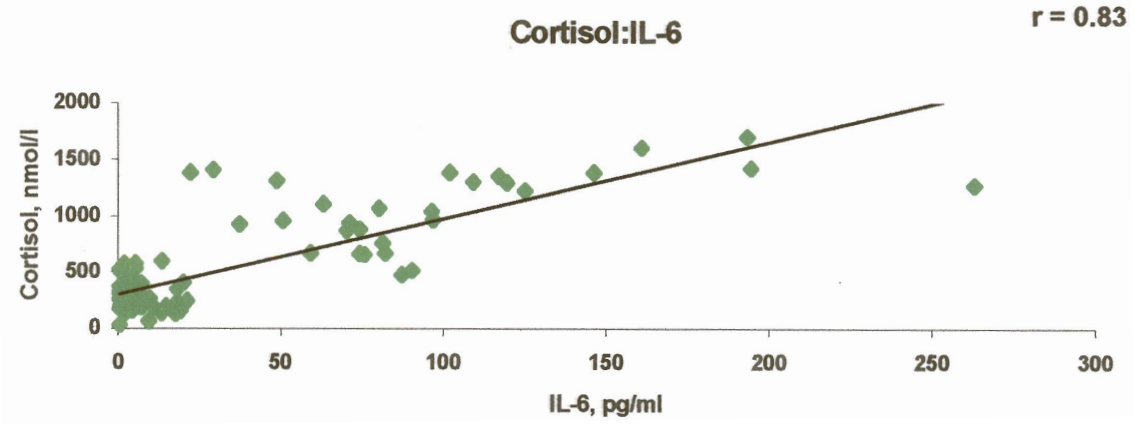

Figure 6.6: The coefficient of correlation between serum cortisol and Il-6

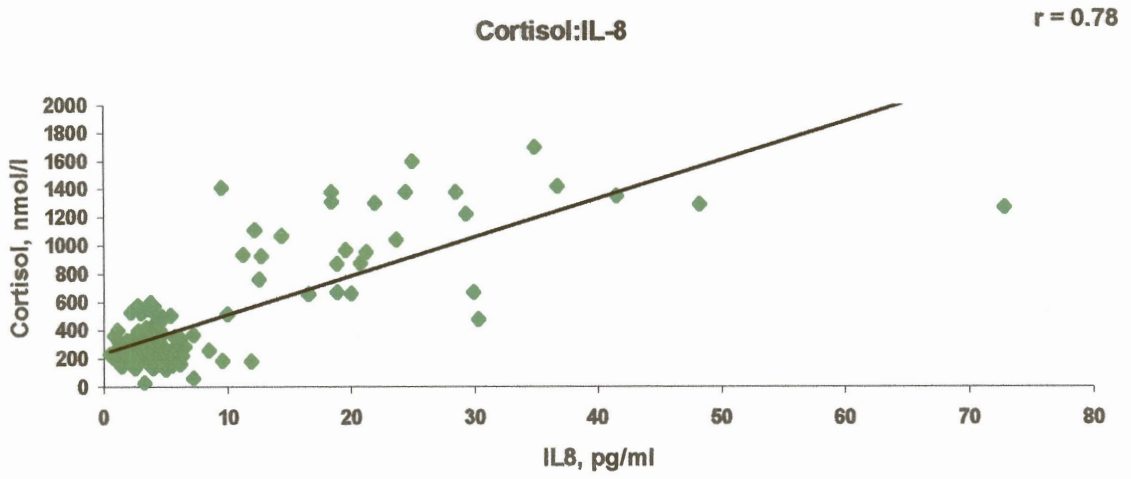

Figure 6.7: The coefficient of correlation between serum cortisol and Il-8

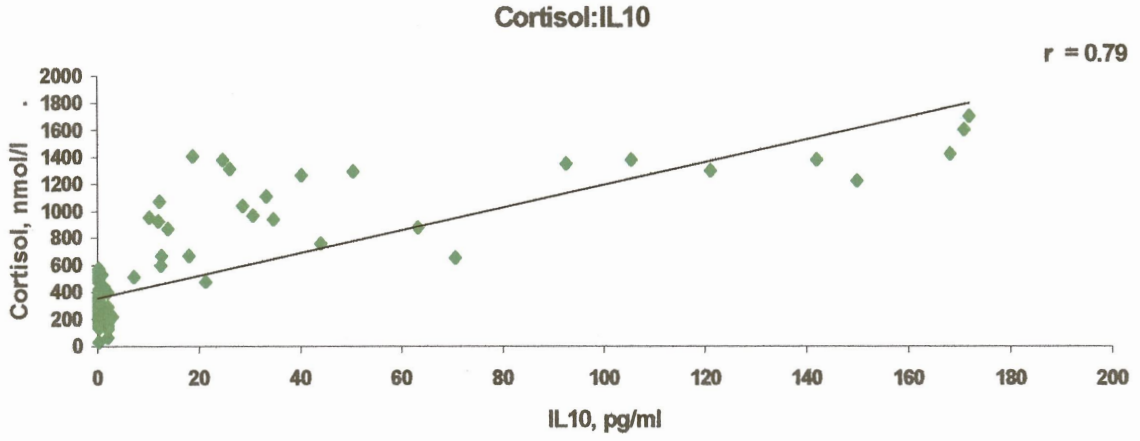

Figure 6.8: The coefficient of correlation between serum cortisol and Il-10

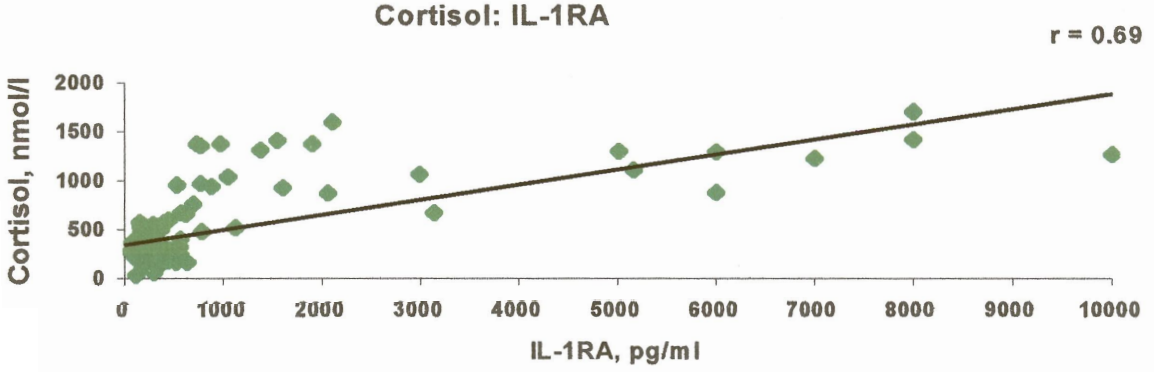

Figure 6.9: The coefficient of correlation between serum cortisol and Il-1RA

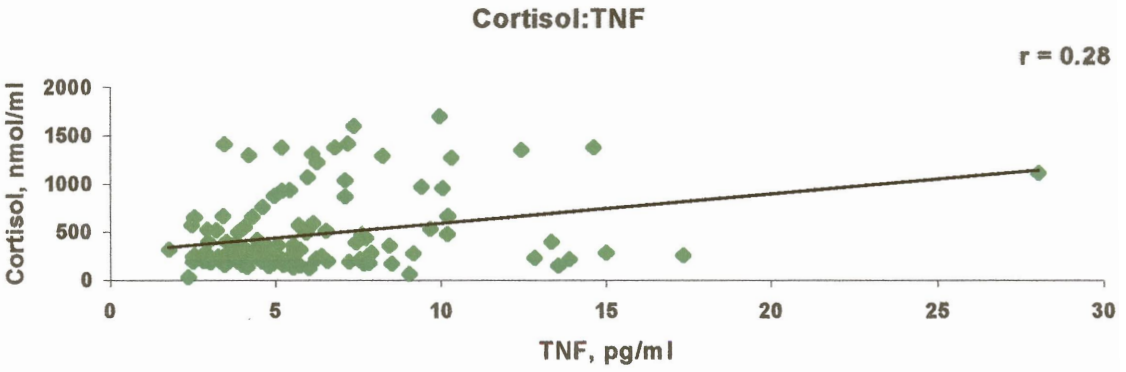

Figure 6.10: The coefficient of correlation between serum cortisol and TNF

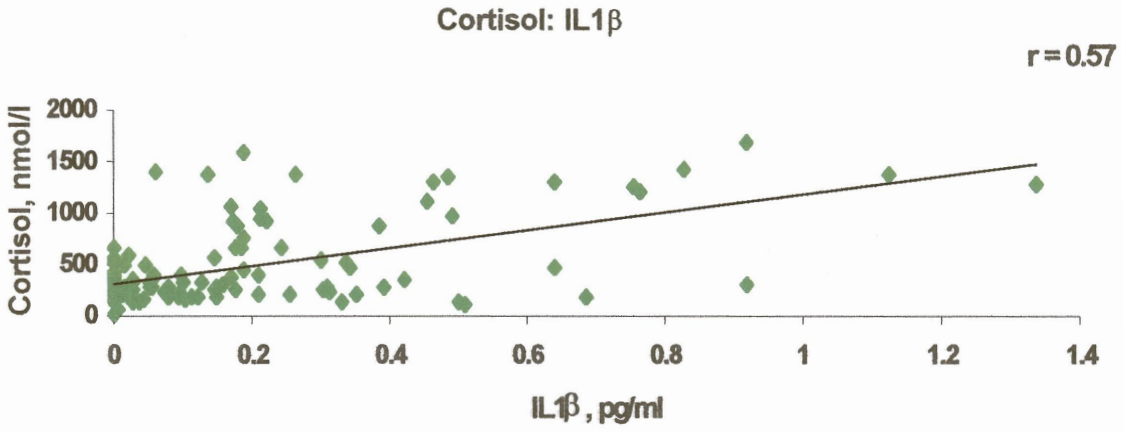

Figure 6.11: The coefficient of correlation between serum cortisol and IL1β

6.4 Discussion

The data from this study and others confirm that plasma concentrations of IL-6, IL-8, IL-10, and IL-1Ra, but not those of TNF-α or IL-1β, rise strongly following prolonged and intensive exertion, falling to near pre-race levels within 24 hours (Drenth *et al.*, 1995; Nehlsen-Cannarella *et al.*, 1997; Ostrowski *et al.*, 1998; Nieman *et al.*, 1998; Suzuki *et al.*, 2000). Strenuous physical exercise of limb muscles typically results in muscle soreness and injury, especially when the exercise is intense and prolonged such as in ultramarathon running. An inflammatory response

to the muscle injury is initiated, characterized by movement of fluid, plasma proteins, and leukocytes into the injured area and metabolically active tissues (Suzuki *et al.*, 2000). Cytokines help regulate the inflammatory cascade, with TNF-α, IL-1β, IL-6, and interferons working synergistically. An exaggerated response is prevented via several pathways, including the production of the anti-inflammatory cytokine, IL-10 and cytokine inhibitor, IL-1Ra, as well as the anti-inflammatory action of cortisol.

The significant reduction in serum cortisol concentrations in the VC-1500 group following the ultramarathon, confirms the results of my earlier study, described in chapter 4. Ostrowski *et al.* (1998b) have provided data supporting the production of IL-6 by neutrophils and macrophages in the area of damaged muscle cells followed by IL-1β and IL-1Ra production by mononuclear cells in the blood compartment. The findings of this work also appear to support this with substantial rises in IL-6 (> 25 fold) and IL-1Ra (>20 fold) being evident following participation in the ultramarathon **(Figures 6.3 and 6.5).** The >5-fold rise in IL-8 concentrations **(Figure 6.2)** supports the work of Suzuki *et al.*, (2000) which showed that this neutrophil chemotactic chemokine is released into circulation after prolonged and intensive, but not moderate exercise.

In the current study the circulating concentrations of IL-1β and TNF-α were only marginally increased post-race, unlike those of IL-6, IL-8 and IL-10. The trivial increases in these cytokines may on the one hand, reflect their relative instability in the circulation as they have been shown to be removed from the circulation rapidly and to later be detected in the urine (Sprenger *et al.*, 1992). It is also possible that peak responses had subsided at the time of sampling (30-45 minutes post-race). Alternatively, complexing to circulating receptors and/or proteolytic degradation may also account for my failure to detect meaningful increases in the circulating concentrations of these two cytokines. The latter contention is supported by reports that both IL-1β and TNF-α are degraded by human neutrophil elastase (Lee & Downey, 2001), a protease, which as previously reported (Gleeson *et al*,1998; Robson *et al.*, 1999) and confirmed in the present study (chapter 7), is elevated in

the circulation during prolonged and intensive exercise, presumably as a consequence of intravascular activation of neutrophils.

Carbohydrate ingestion has been reported to attenuate post-exercise increases in cortisol, IL-6, IL-1Ra, and IL-10 by maintaining blood glucose levels (Nieman *et al.*, 1998). In the present study, daily supplementation with 1500 mg, but not 500 mg of vitamin C during the week prior to the race was also associated with lower post-exercise concentrations of cortisol and the anti-inflammatory cytokines, IL-1Ra and IL-10. This effect was not confounded by race day intake of carbohydrate, with all groups ingesting large, but comparable, quantities due to the extreme conditions of the Comrades Ultramarathon race. Pre-race serum vitamin C correlated negatively, but weakly, with post-race serum cortisol levels, while post-race serum cortisol correlated positively with post-race plasma Il-10, IL-6, IL-1β and IL-1Ra levels. When pre- and post-race data were pooled (N=124), correlation co-efficients between serum cortisol and the polypeptides measured, exceeded 0.68 for all but TNF and IL-1β **(Figures 6.6-6.11)**.

These high correlations lend support to the previously described interactions between pro-inflammatory cytokines, cortisol and the anti-inflammatory polypeptide cascade (Mandrup-Poulsen *et al.*, 1995). While glucocorticoid release has been shown to be activated by the pro-inflammatory cytokines, IL-6 and IL-8 (Figure 2.4), the anti-inflammatory cytokine, IL-10, which induces the production of cytokine inhibitor, IL-1Ra, is well known to respond to increased circulating cortisol concentrations. The apparent attenuation of IL-6, IL-8, IL-10 and IL-1Ra in the post-race samples of the VC-1500 group therefore confirms a possible link between these cytokines and the significantly lower serum cortisol concentrations following prolonged and intensive exertion. In this study, the association is particularly significant in terms of IL-10, IL-1Ra and cortisol. Possible reasons for this, which may well relate to inhibition of the activation of the pro-inflammatory transcription factors, have been described in chapter 5.

Another mechanism may explain the association between vitamin C supplementation and the reduced post-race cytokine levels observed in the VC-1500 group. During exercise, the increase in oxygen uptake by active muscles causes an increase in the generation of reactive oxygen species (ROS). Strenuous exercise also causes an influx of neutrophils into muscle tissues which are considered to be one of the main sources of extracellular ROS (Peters, 1997). ROS cause a wide spectrum of cellular damage and may mediate leukocyte apoptosis (Azenabor & Hoffman-Goetz, 1999; Campbell *et al.*, 1999). As discussed in chapter 2, the majority of studies have indicated that vitamin C supplementation does not completely prevent, but attenuates exercise-induced oxidative stress. This has been confirmed by increases in the levels of serum diene conjugation (Vasankari *et al.*, 1998), thiobarituric acid reactive substances (Rokitzki *et al.*, 1994b), malondialdehyde and exhaled pentane (Kanter et al., 1993; Ashton *et al.* ,1999; Thompson *et al.*, 2001b), the oxidation of low-density lipoproteins (Sanchez-Quesada *et al.*, 1998; Ashton *et al.*, 1999) and electron spin resonance (Ashton *et al.* ,1999). There is growing evidence that by protecting cells from oxidative damage, inflammation and cytokine production may be reduced by vitamin C supplementation (Grimble, 1997; Chen et al., 1998; Schwager & Schulze, 1998b; Bijur, 1999). It has also been reported that vitamin C prevents glucocorticoid-induced apotosis in murine lympocytes (Campbell *et al.*, 1999).

As neutrophil ROS have damaging effects on the neutrophils themselves (**Figure 2.3**), these cells acquire a high level of ascorbic acid for protective purposes (Wolf, 1993). As it has been shown that Vitamin C supplementation can attenuate neutrophil oxygen radical production (Dwenger *et al.*, 1992), it is possible that a mechanism by which vitamin C supplementation prior to prolonged and intensive exercise may lower post-race cytokine levels, is by reducing neutrophil oxidative stress on muscle cells (Goldfarb, 1999).

In summary, runners completing the 90 km Comrades Marathon experienced substantial increases in concentrations of serum cortisol and plasma IL-6, IL-8, IL-10 and IL-1Ra. Although these increases were significantly attenuated in runners

ingesting ≥1500 mg, but not ≤500 mg vitamin C supplements for one week prior to the race and on race day, and these attenuations reached statistical significance only in the cases of IL-10 and IL-1Ra, a larger scale study with greater sample sizes is required to establish whether the trends towards possible attenuation of post-race IL-6 and IL-8 ($p = 0.11$; 0.14) were meaningful and could reach statistical significance.

An interesting novel finding in this paper was the trend towards the higher circulating concentrations of the inflammatory cytokine, IL-6, and the chemotactic cytokine, IL-8, in the group ingesting 500 mg vitamin C. In the next chapter, the question of muscle inflammation is investigated in more depth and the cytokine response in the three groups is integrated with that of acute phase proteins and elastase, a marker of neutrophil degranulation.
